

In Vitro Effect of Cyclic Adenosine 3', 5'-Monophosphate (cAMP) on Early Human Ovarian Follicles

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Purpose: To test the effect of cyclic adenosine 3', 5'-monophosphate (cAMP) on early human ovarian follicles during prolonged culture period.

Methods: Donated ovarian biopsies from 16 women undergoing gynecological laparoscopy were cut into slices and cultured in parallel for 1, 2, or 3 weeks in the presence and the absence of 0.5 mM 8-bromo-cAMP. The developmental stages, sizes, and viability of the follicles were recorded from histological sections of all samples.

Results: On day 14, cortical slices cultured with 8-bromo-cAMP showed a significantly higher proportion of secondary follicles (50.0% vs. 20.0%) and a lower proportion of primordial follicles (9.7% vs. 26.7%) when compared with those cultured without 8-bromo-cAMP. On day 21, the proportion of viable follicles in cortical slices with 8-bromo-cAMP treatment was significantly higher than that without 8-bromo-cAMP treatment (79.6% vs. 55.2%).

Conclusion: CyclicAMP promoted folliculogenesis and follicle survival during 14–21 days' culture of human ovarian cortical slices.

KEY WORDS: cAMP; human; organ culture; ovarian follicles.

INTRODUCTION

Obtaining mature human oocytes from cultured primordial, primary, and secondary ovarian follicles would benefit women who have their ovarian tissue cryopreserved because of cancer or certain genetic disorders (1). Human primordial and primary ovarian follicles have been successfully cultured to secondary and occasionally to antral stages within slices of ovarian cortical tissue (2–6). In these cultures, follicle stimulating hormone (FSH) (2), insulin, insulin-like

growth factors I and II (4), and growth differentiation factor-9 (6) have had positive effects on the growth and survival of the follicles.

Cyclic adenosine 3', 5'-monophosphate (cAMP) is an intracellular second messenger and regulates a number of cellular processes, such as cell growth and differentiation, ion channel conductivity, neurotransmitter release, and gene transcription (7,8). The cAMP system is an important regulator of ovarian hormone secretion, growth factor production, follicular apoptosis, and oogenesis (9,10).

The effects of cAMP on later stages of follicular development have been shown in animal experiments. Dibutyl cyclic AMP (dcAMP) stimulates the growth of mouse preantral and antral follicles in culture (11). In rat, dcAMP suppresses the apoptosis of early antral follicles in vitro (12). 8-Bromo-cAMP and forskolin, both cAMP analogues, promote the growth of mouse preantral follicles (13).

The results of several animal experiments also suggest an important role of cAMP in early follicular

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development. Dibutyl cyclic AMP increases expression of the gene encoding kit ligand (also called stem cell factor) in mouse granulosa cells (14). Kit ligand is considered to be a necessary factor involved in the initiation of follicular growth and early follicular development (15,16). Cyclic AMP induces formation of the FSH receptor (17,18) and stimulates the synthesis of aromatase in rat primordial follicles (19).

We tested whether 8-bromo-cAMP (one of the cAMP analogues) can improve the development and survival of early human ovarian follicles during prolonged culture period.

MATERIALS AND METHODS

The study was approved by the Research Ethics Committees of the Department of Obstetrics and Gynecology, Helsinki University Central Hospital, the Family Federation of Finland, and the Karolinska Institute, Huddinge University Hospital.

Donated ovarian cortical biopsies, about $2 \times 5 \times 5$ mm in size, were obtained from 16 women undergoing gynecological laparoscopy, after informed consent. The age (Mean \pm SD) of the women was 31.5 ± 4 (range, 25–43) years.

Each ovarian biopsy was placed in HEPES-buffered culture medium (MEM, Gibco BRL, Life Technologies, Paisley, Scotland) and transported to the laboratory. After most of medullar tissue had been removed, the remaining cortex was cut into slices of 1–2 mm³. These tissue slices were randomly divided into seven groups. One group was fixed directly before culture. The others were cultured in parallel in the presence and the absence of 0.5 mM 8-bromo-cAMP (Calbiochem, CA) for 1, 2, or 3 weeks.

The cortical slices were cultured as described earlier (3,5). Briefly, the tissue slices were cultured on precoated Millicell CM inserts (12-mm diameter, 0.4- μ m pore size; Millipore, Bedford, MA) sitting in 24-well plates (Nunc, Roskilde, Denmark). The inserts were precoated with extracellular matrix (Matrigel, Becton Dickinson, MA), 100 μ L per insert, at a dilution of 1:3 in serum-free culture medium. Culture medium was added, 100 μ L inside the insert and 400 μ L outside the inserts in the well. The plates were placed in a humidified incubator at 37°C with 5% CO₂. The culture medium was changed every second day.

The culture medium was Earle's balanced salt solution (Gibco BRL, Life Technologies, Paisley,

Scotland) supplemented with pyruvate (0.47 mM, Sigma, St. Louis, USA), antibiotic/antimycotic solution (50 U penicillin G/mL, 50 μ g streptomycin sulfate/mL, 0.125- μ g amphotericin B/mL, Gibco, Paisley, Scotland) and 5% heat-inactivated human serum obtained from women undergoing pituitary desensitization for in vitro fertilization. The serum was taken after 14 days of downregulation with GnRH agonist (Buserelin, Suprecur; Hoechst, Frankfurt-am-Main, Germany).

The tissue pieces were harvested at different time points. After fixation in Bouin's solution, they were dehydrated and embedded in paraffin. The tissue was cut into two-micron serial sections and stained with hematoxylin and eosin.

Developmental stages of the follicle were classified by the shape and number of the granulosa cells surrounding the oocyte. We defined primordial follicles as those containing one layer of flat granulosa cells, primary follicles as those with one layer of granulosa cells containing one or more cuboidal granulosa cells, and secondary follicles as those containing two or more layers of cuboidal granulosa cells at least at one site of the follicle.

Eosinophilia of the ooplasm, contraction and clumping of the chromatin material and wrinkling of the nuclear membrane of the oocyte and/or presence of pyknotic granulosa cells were regarded as signs of follicle atresia (20). To avoid double counting of follicles, at least 10 sections were omitted between analyzed sections. Two to three adjacent sections were also examined to observe more than one section of the same follicle. Only follicles with the oocyte nucleus visible were counted.

To measure the diameters of oocytes and follicles, we used a digital image analysis system (Easy Image Mätning, Bergström Instruments, Stockholm, Sweden) connected to an inverted microscope (Nikon, Bergström Instruments, Stockholm, Sweden). The diameters of the oocytes and follicles were measured only in those with oocytes nuclei visible.

Statistical Analyses

The chi-square test was used to analyze differences in developmental stages and the proportions of viable follicles between the groups. Fisher's exact test was used where appropriate. Student's *t* test was used for analyses of differences in the diameters of the follicles and oocytes. *P* < 0.05 was considered statistically significant.

Table I. Developmental Stages of Follicles in Cortical Slices Before and After Culture

	Primordial		Primary		Secondary		Atretic	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Before culture	73	69.5	24	22.9	8	7.6	0	0
Cultured without cAMP on day 7	12	21.81*	17	30.9	26	47.3*	19	24.7
Cultured with cAMP on day 7	14	20.6*	24	35.3	30	44.1*	15	17.4
Cultured without cAMP on day 14	16	26.7	32	53.3	12	20.0	14	18.7
Cultured with cAMP on day 14	6	9.7**	25	40.3	31	50.0***	13	17.1
Cultured without cAMP on day 21	4	26.7	6	40.0	5	33.3	13	44.8
Cultured with cAMP on day 21	6	13.0	17	37.0	23	50.0	11	20.4

* $p < 0.001$, compared with that before culture; ** $p < 0.05$, *** $p < 0.001$, compared with control (without cAMP).

RESULTS

First Week of Culture

The majority of follicles initiated their growth during the first 7 days of culture regardless of the supplement used. In fresh cortical slices before culture, most of the follicles were at the primordial stage and only a small proportion of follicles were secondary. After 7 days of culture, the proportions of primordial follicles decreased dramatically (from 70% to 21–22%), while those of secondary follicles had increased significantly (from 8% to 44–47%) in all cultured slices, compared with those in fresh slices before culture (all $P < 0.001$; Table I, Fig. 1). The proportions of live follicles decreased significantly by day 7 in both cultures with and without cAMP compared with those before culture (from 100% to 75–83%, both $P < 0.001$; Fig. 2).

On day 7, in all slices cultured with and without 8-bromo-cAMP, the follicular diameters had increased

significantly compared with those before culture (from 41 μm to 48–52 μm , both $P < 0.001$), while the diameters of the oocytes did not change as much (32–34 μm ; Fig. 3).

There were no significant differences between cultures with and without 8-bromo-cAMP regarding to the proportion of follicles at different stages, the percentage of live follicles, and the diameters of follicles and oocytes. Hence, after 7 days culture, 8-bromo-cAMP did not show any significant effect on follicular growth or survival.

Second and Third Weeks of Culture

On day 14, cortical slices cultured with 8-bromo-cAMP showed a significantly higher proportion of secondary follicles (50.0% vs. 20.0%, $P < 0.001$) and a lower proportion of primordial follicles (9.7% vs.

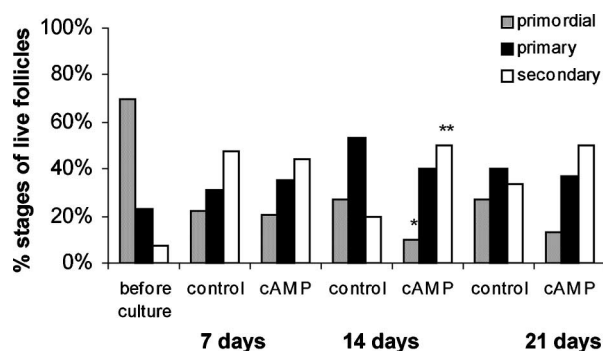


Fig. 1. Proportions of primordial, primary, and secondary follicles before culture and after 7, 14, and 21 days of culture with and without 8-bromo-cAMP (control). The asterisks mark a lower proportion of primordial follicles (* $P < 0.05$) and a higher proportion of secondary follicles (** $P < 0.001$) after 14 days of culture with 8-bromo-cAMP, compared with control culture.

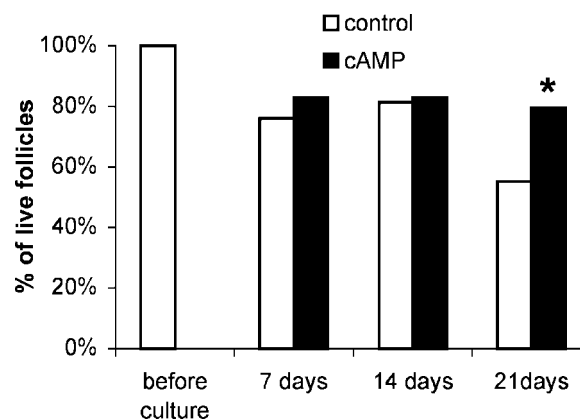


Fig. 2. The proportion of live follicles before culture and after culture in the presence and the absence (control) of 8-bromo-cAMP. The proportions of viable follicles in cultures had decreased significantly by day 7 compared with those before culture (both $P < 0.001$). The asterisk marks a significantly higher proportion of live follicles after 21 days of culture with 8-bromo-cAMP compared with control culture ($P < 0.05$).

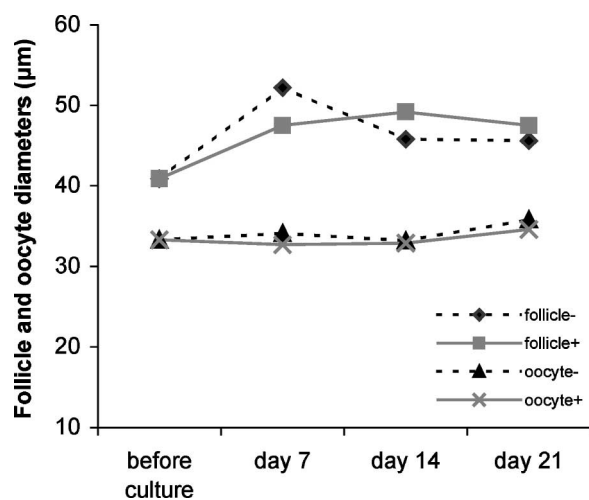


Fig. 3. Mean diameters of follicles and oocytes before culture and after culture with (follicle+, oocyte+) and without (follicle-, oocyte-) 8-bromo-cAMP. All follicles after culture were significantly larger than those before culture independent of supplement used (all $P < 0.001$). The oocytes did not grow significantly during culture.

26.7%, $P < 0.05$) when compared with those cultured without 8-bromo-cAMP (Table I, Fig. 1). During 14 days of culture, the presence of 8-bromo-cAMP in the culture medium did not increase the proportion of live follicles. The follicles in all cultures on day 14 were larger than those before culture ($P < 0.001$); while the size of the oocytes remained almost unchanged (Fig. 3). No significant differences in the diameters of the follicles or oocytes between the cultures with and without 8-bromo-cAMP were found. A secondary follicle after 14 days of culture with 8-bromo-cAMP was shown in Fig. 4.

After 21 days of culture, there were no significant differences in the developmental stages of the follicles or the diameters of follicles and oocytes between cortical slices cultured with and without 8-bromo-cAMP (Figs. 1 and 3). However, on day 21, the proportion of live follicles in cortical slices with 8-bromo-cAMP treatment was significantly higher than that without (79.6% vs. 55.2%, $P < 0.05$; Fig. 2).

DISCUSSION

During the first 7 days of culture, cAMP did not show any significant effect on follicular growth and survival. But after 7 days, it had resulted in enhanced folliculogenesis and the effect was clearest by day 14. 8-Bromo-cAMP also acted as a survival factor during culture, with a significant effect observed by day 21.

In human, the oocyte enters the growth phase when it becomes completely enclosed by approximately 15 cuboidal granulosa cells (21). The oocytes begin to grow when the follicles are at late primary or early secondary stage. In our study, most secondary follicles were at an early stage during culture, which explains why the oocytes did not grow significantly. Similar results are obtained by de Bruin *et al.* (22). They find that the sizes of oocytes do not change with the developmental stage before the primary stage.

The serum applied in our experiment was not completely free of FSH. Since the blood sample was taken from patients undergoing in vitro fertilization after 14 days of downregulation, FSH concentration in the serum was very low and it decreased further when serum was diluted 20 times in the culture medium. FSH in the culture medium hardly had any effect on cultured follicles. 8-Bromo-cAMP is the only variable between treated groups. Therefore, the improved folliculogenesis and follicle survival observed in our study must be due to 8-bromo-cAMP itself.

Ovarian biopsies from the young healthy women with high density of follicles are very difficult to obtain. That is why we observed only a small number of follicles even from 16 patients. According to power analyses (with power = 0.8), the numbers of the follicles we have presented in the study are adequate.

The density and developmental stages of follicles in human ovarian cortex do vary both between and within individuals. In our case, the biopsy was quite small ($2 \times 5 \times 5$ mm) and was cut into even smaller slices ($1-2$ mm³) for random distribution into different groups. The material variation between different groups was very limited. Furthermore, we compared the proportions, not the absolute follicle numbers. We do not think the limited variation in the absolute follicle numbers between groups could influence the conclusion we draw from the study.

The effects of cAMP on follicle development in the present study were in accordance with those obtained by using FSH supplementation (2). FSH supplementation did not increase the diameters of the follicles or promote the developmental stages during the first 5 days of culture. The proportion of primordial follicles had decreased dramatically in both control and FSH-treated cultures after 5–7 days. However, on day 15, the follicles cultured with FSH were significantly larger than those cultured without FSH. During 5–15 days of culture, FSH acted as a survival factor, with a significantly

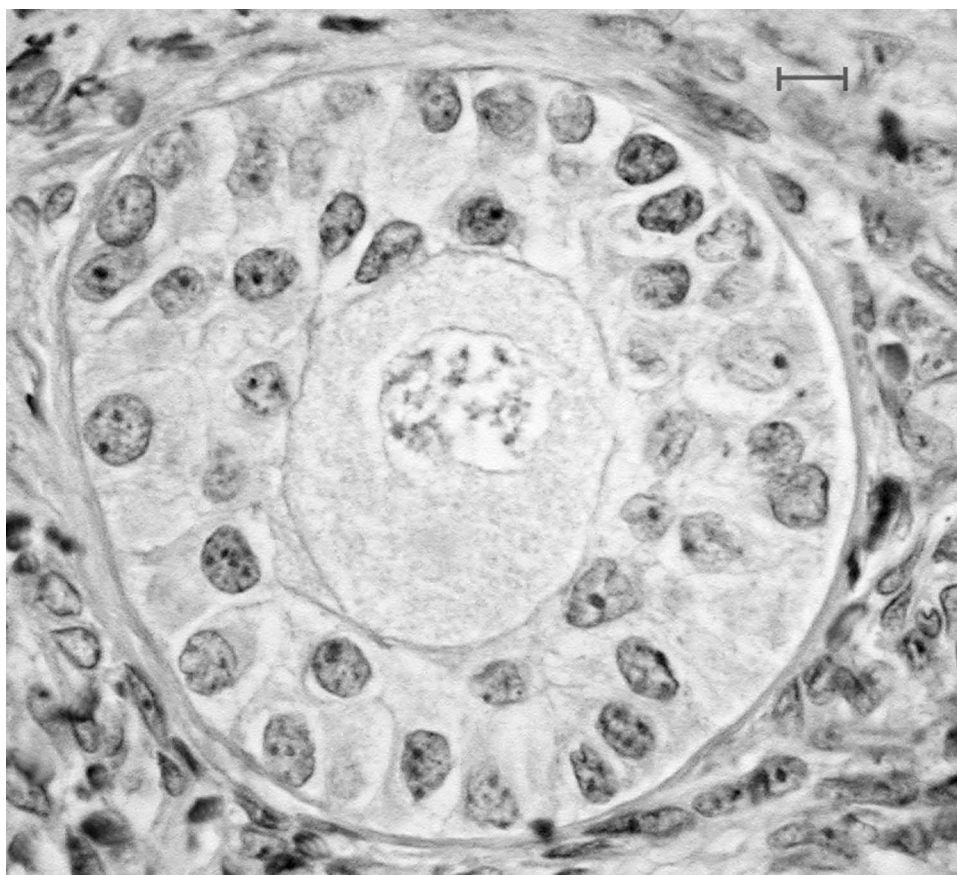


Fig. 4. A secondary follicle after 14 days of culture with 8-bromo-cAMP. The diameters of the follicle and oocyte were 95.3 and 50.0 μm respectively. Bar = 0.7 μm .

reduced proportion of atretic follicles. These observations, taken together with the present results, indicate that both cAMP and FSH promoted folliculogenesis and follicle survival during 2 to 3 weeks culture. It would be interesting to test combined effect of cAMP and FSH on the early human ovarian follicles.

FSH is involved in early folliculogenesis in human (2). In human, 50% of follicles at the primary stage express FSH receptor (23). Women with an inactivating mutation in the FSH receptor are infertile as they have only primordial, primary, and occasionally secondary follicles (24). How to completely overcome FSH receptor inactivation in culture and obtain mature oocytes for assisted reproduction for these women remains a challenge. Cyclic AMP is the second messenger of FSH. FSH exerts its effect on granulosa cells partly through the receptor-activated cAMP-PKA (protein kinase A) pathway. Cyclic AMP might replace at least some FSH action of early follicular development as our results showed. Culture

of follicles with 8-bromo-cAMP followed by in vitro maturation of the oocytes may facilitate overcoming FSH receptor inactivation.

Our results indicate that cAMP has effects on the early human ovarian follicles cultured in cortical slices by promoting folliculogenesis and follicle survival at 14 and 21 days. We had not observed the effect of 8-bromo-cAMP on follicles at late stages from our study, because there were few preantral and antral follicles in the ovarian cortical biopsies. It is possible to test the effect of 8-bromo-cAMP on follicles at late stages by culturing them individually after isolation. We do have plans to conduct these studies sometime in the future.

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